# SELECTIVITY OF PIEZOELECTRIC-EXCITED MILLIMETER-SIZED CANTILEVER (PEMC) SENSORS TO *BACILLUS ANTHRACIS* SPORES IN THE PRESENCE OF *BACILLUS THURINGIENSIS* AND *BACILLUS CEREUS* SPORES IN A FLOW CELL

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## ABSTRACT

We report a highly sensitive, rapid and reliable method for the detection of *Bacillus Anthracis* spores at 300/mL using a piezoelectric-excited millimeter-sized cantilever (PEMC) sensor. Antibody specific to *Bacillus anthracis* (BA, Sterne strain 7702) spores was immobilized on PEMC sensors, and exposed to spores (300 to $3x10^6$ spores/mL). The resonant frequency decreased at a rate proportional to the spore concentration and reached a steady state frequency change of 5 ± 5 (n=3), 92 ± 7 (n=3), 500 ± 10 (n=3), 1030 ± 10 (n=2), and 2696 ± 6 (n=2) Hz corresponding to 0, $3x10^2$, $3x10^3$, $3x10^4$, and $3x10^6$ spores/mL, respectively. Selectivity of the antibody functionalized sensor was determined with samples containing BA (3 x $10^6$/mL) mixed with *Bacillus thuringiensis* (BT; $1.5x10^9$/mL) in various volume ratios that yielded BA:BT ratios of 1:0, 1:0.008, 1:0.004, 1:0.002 and 0:1. The corresponding resonance frequency decreases were, 2345, 1980, 1310, 704 and 10 Hz. Sample containing 100% BT spores (1.5 x $10^9$/mL and no BA) gave a steady state frequency decrease of 10 Hz, which is within noise level of the sensor, indicating excellent selectivity. These results show that detection of *Bacillus anthracis* spore at a very low concentration (300 spores/mL) and with high selectivity in presence of another *Bacillus* spore (BT) can be accomplished using piezoelectric-excited millimeter-sized cantilever sensors.

## INTRODUCTION

There is an urgent need for biosensors that detect *Bacillus anthracis* spores rapidly in a highly selective and sensitive fashion. In response to the *anthrax* threat, various detection techniques capable of providing reliable identification of *anthrax* spores are currently under development. These include, evanescent wave fiber-optic biosensors[1], real-time PCR[2,3], and quartz crystal microbalance (QCM)[4]. When confronted with the requirement of low detection limit, these sensor platforms fall short. Traditionally, the micro-organisms are grown on selective media for at least 24 h, followed by morphological and biochemical analysis[5,6]. The 24-hour incubation time is for too limiting, particularly in the context of public safety. Hence, there is a need for a simple and inexpensive method for the detection of *Bacillus anthracis* spores in real time. Furthermore, the United State Postal Service (USPS) has required the development of a rapid detection method, which is cost effective, for the identification of bioterrorism threat agents[7]. In a similar manner the Department of Transportation (DOT) requires a detection system that will identify bio-terrorism agents within 20 minutes of exposure.

In this paper, we present the piezoelectric-excited millimeter-sized cantilever (PEMC) sensors for the continuous detection of *anthrax*-causing *Bacillus anthracis* spores at a concentration as low as 300 spores/mL in liquid buffer. Millimeter-sized cantilever sensors are suitable for biologics detection due to their high performance characteristics: high sensitivity, short response time, robustness, selectivity, resonance stability, and surface regeneration capabilities. It is worth noting that a complete biosensor system for the control and prevention of a bioterrorism attack requires a three-step process: a detection step, an identification step, and finally a communication step[8]. Here, we employ the PEMC sensor as a first step detector.

## SENSOR PHYSICS

The physics of PEMC sensor can be found elsewhere[9]. The resonant frequency change is a direct function of the cantilever's mass and can be expressed as $\left(f_{nf} - f_{nf}^{'}\right) = \frac{1}{2} f_{nf} \frac{\Delta m}{M_e^{'}}$, where $(f_{nf} - f_{nf}^{'})$ is the change in resonant frequency of the $n^{th}$ mode in fluid due to mass of pathogen attached, $\Delta m$. $M_e^{'}$ is the effective mass of the cantilever under liquid immersion condition.

## FABRICATION

The PEMC sensors were manually fabricated as a composite structure of two layers: a 127μm thick lead zirconate titanate (PZT) layer (Piezo Systems Inc., Cambridge, MA) and a 160μm thick glass (Fisher Scientific). The cantilever was 3.5 mm long and 1 mm wide. The two layers were bonded so that 2 mm of glass overhangs the PZT. The overhang glass (2 mm) provides surface for antibody immobilization and pathogen detection.

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## MATERIALS AND METHODS

*Bacillus anthracis* spores, the Sterne strain 7702, and Protein A purified Rabbit polyclonal antibody (rPC) in PBS was provided by Professor Richard Rest (Drexel University College of Medicine). An *anthrax* combo Goat polyclonal antibody (gPC; Catalog designation: AB8301) raised against a gamma inactivated spore mixture of Ames, Sterne, and Vollum strains was purchased from Chemicon International (Temecula, CA). All other chemical reagents were from Sigma-Aldrich.

The sensing glass surface was cleaned and then, aminated with 0.4% 3-aminopropyl-triethoxysilane (APTES) in deionized water at pH 3.0. EDC/sulfo-NHS chemistry was used to catalyze the reaction between antibody and amine groups on the cantilever surface. Spore samples in concentrations ranging from 3 x $10^4$ to 300 spores/mL were prepared in phosphate buffered saline (PBS) (10 mM, pH 7.4) by serial dilution of the master sample (3 x $10^6$ spores/mL). The selectivity of the PEMC sensors was investigated using samples prepared by mixing *Bacillus anthracis* spores and *Bacillus thuringiensis* (BT) spores. *Bacillus thuringiensis* spore powder was purchased from EDVOTEK (West Bethesda, MD) and a stock solution of 1.5 x $10^9$ spores/mL was prepared in PBS solution, pH 7.4. The mixed samples were prepared in various volume ratios stock solutions of *Bacillus anthracis* (3 x $10^6$/mL) and *Bacillus thuringiensis* spores (1.5 x $10^9$/mL) to yield spore concentrations labeled A, B, C, D and E whose BA concentration was 100%, 0.79%, 0.40%, 0.20% and 0 %. The detection experiments were carried out in a chamber at a temperature of 22 ± 0.5 $^o$C.

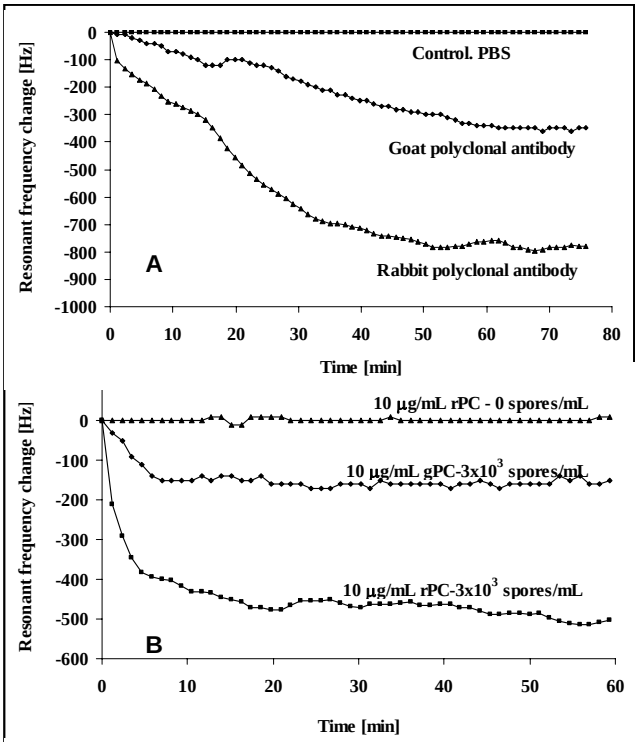


**Figure 1**: **Panel A:** Resonant frequency change due to the binding antibodies. **Panel B:** The binding of spores (3,000 spores/mL) to the antibodies on the sensor surface.

## RESULTS AND DISCUSSION

### Resonance characterization of PEMC sensors

Three cantilevers were fabricated and used in the *Bacillus anthracis* spores detection experiments. For brevity and comparison of results for the various spore concentration results, only the data from one cantilever is presented here. Each experiment was repeated at least twice and the data shown are typical of the results obtained. The cantilever sensor used has fundamental and second bending mode resonant frequencies of 14 ± 0.05 kHz and 62 ± 0.05 kHz. Several repeated experiments showed that these resonant frequencies are stable within ± 50 Hz. In any one experiment, the fluctuation of the steady state resonant frequency was at most ± 10 Hz. In this study, the second mode was selected for in-liquid detection experiments because the sensitivity of a resonant mode is directly proportional to the resonant frequency and therefore, the second mode is of a higher sensitivity.

### Selection of Antibody for the detection of Bacillus anthracis spores

In order to select a suitable antibody for BA detection, several experiments were carried out under the same conditions using the two polyclonal antibodies, gPC and rPC. PEMC sensors were functionalized with APTES as previously described and then exposed to 10 μg/mL antibody solution at 22 $^o$C in a batch measurement cell. The results shown in Figure 1 (Panel A) is typical of the second flexural mode resonant frequency change for the amide bond formation between the aminated sensor surface and the activated carboxylic groups on the antibody. The resonant frequency decreased exponentially, indicating rapid initial reaction and reaches a steady state value suggesting an equilibrium surface coverage. Also, the steady state resonant frequency change was greater for the rPC (780 Hz) compared to gPC (350 Hz), suggesting that a larger mass of rPC was immobilized. In Figure 1 (Panel B), the results of resonant frequency response to the binding of spores (3,000 spores/mL) to the sensor surfaces are shown. It can be seen that the equilibrium resonant frequency change obtained with the rPC antibody is 510 Hz compared to 165 Hz obtained with gPC antibody. Similar experiments conducted with 30,000 spores/mL (data not shown) resulted in 1040 Hz change with rPC

compared with 510 Hz with gPC. Greater spore attachment with larger amount of antibody immobilized on sensor surface is an expected result. The reason why Rabbit polyclonal antibody was immobilized to a greater extent compared to gPC was not further investigated. We assumed that the concentration of the antibody provided by the suppliers were as given; no antibody assays were carried out. Since a larger response was obtained with rPC antibody, we used it in all further experiments reported in this paper.

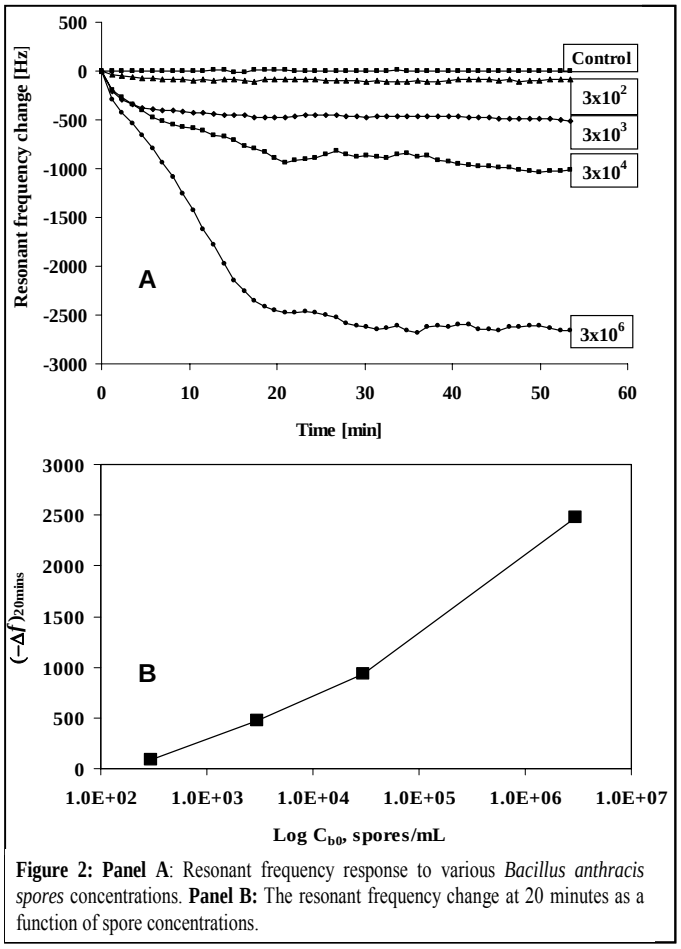


**Figure 2: Panel A**: Resonant frequency response to various *Bacillus anthracis spores* concentrations. **Panel B:** The resonant frequency change at 20 minutes as a function of spore concentrations.

## PEMC Sensor Response to Spores of various concentrations

Figure 2 (Panel A) shows the time profile of the second mode resonant frequency change of PEMCa antibody-functionalized sensor in the detection of *Bacillus anthracis* spores at concentrations of $3x10^2$, $3x10^3$, $3x10^4$ and $3x10^6$ spores/mL. In all cases, the response showed a rapid decrease during the first 5 to 15 minutes followed by a slower change reaching a constant resonant frequency. For the highest spore sample ($3x10^6$ spores/mL), the rate of decrease was more rapid compared to the lower concentration (300 spores/mL) sample. This is an expected response, as the binding rate is proportional to concentration. The total change was $2696 \pm 6$ Hz (n=2) for 3 x $10^6$/mL sample and $92 \pm 7$ Hz (n=3) for the 300 spores/mL sample. Several control experiments with antibody-functionalized sensor were conducted in PBS solution for 1 h at the same experimental conditions as was used in the detection experiments. The response, included in Figure 2, showed fluctuations in the steady state resonant frequency of ± 5 Hz, which is also the sweep frequency step size. That is, the fluctuations are likely to be even lower than this value. When one compares this with the response of 300 spores/mL sample, it is clear that steady state response to noise in measurement was in the order of 20 for the low spore count sample. For higher spore concentration samples, the steady state change in resonant frequency was significantly higher than measurement errors in resonant frequency. The observed resonant frequency fluctuation of ± 5 Hz for the final steady state value indicates that the cantilever resonance characteristic is quite stable under liquid immersion.

One notes that in Figure 1 the total change in the resonant frequency for the antibody immobilization was greater than the steady state resonant frequency change obtained for the corresponding spore attachment. One possible explanation is that the antibody forms a monolayer on the sensor surface due to its smaller size compared to spores. Under liquid immersion conditions, liquid is readily entrained within the proteinous layer contributing to the oscillating mass. On the other hand, spore attachment was not dense, and was scattered and distributed. Such a low density surface coverage is not conducive to entrapping liquid mass, and thus a lower frequency change was observed. This interesting aspect of changes in the interfacial characteristics is currently under study, and is not within the scope the current paper.

The mass change sensitivity of the sensor was determined using the silicone oil dip touch technique that we reported earlier[10]. The mass change sensitivity under liquid immersion was determined as 1.56 x $10^{-11}$ g/Hz. Using the mass change sensitivity, the mass of *Bacillus anthracis* spore bound to the sensor surface after one hour from the lowest to the highest spore concentrations were, respectively, $1.2 \pm 0.05$ng, $7.8 \pm 0.10$ ng, $16.2 \pm 0.10$ ng and $42.1 \pm 1.50$ ng.

The sensing requirement for bioterrorism agents suggested by several US Federal Agencies (for example, Homeland Security Advanced Research Projects Agency) is 20 minutes detection time after exposure to bioterrorism agents. Hence it is useful to examine the results of our experiments in the context of this time frame. In Figure 2, Panel B, a plot of resonant frequency change following 20 minute exposure of sensor versus the log of spore concentration. Resonant frequency change increases in a nearly linear fashion with spore concentration, in the concentration range investigated. These results suggest that calibration relationships for estimating spore concentration can be stated as:

$$\log(C_{b0}) = \frac{(-\Delta f)_{20\,\min} + A}{B}$$

Where A is y-intercept and B is the slope of the line Fig 2, Panel B. The parameters A and B will depend on cantilever dimensions, antibody type, and immobilization method.

The inset in Fig. 2B shows an ESEM photomicrograph of the cantilever surface after the detection of 3 x $10^6$ spores/mL for 1 h. About ten fields of 20 to 1280 $\mu m^2$ area were examined, and the photograph is typical of the high surface density region. Spore surface density was not uniform when viewed at 20 $\mu m^2$ area at 25,000X, but appeared uniform at lower resolution in the high surface density region, an area of 33 $\mu m^2$ viewed at 15,000X. In none of the samples we examined, close packing of cells on sensor surface was observed. It is estimated that sensor surface covered by spores was always less than 20 %.

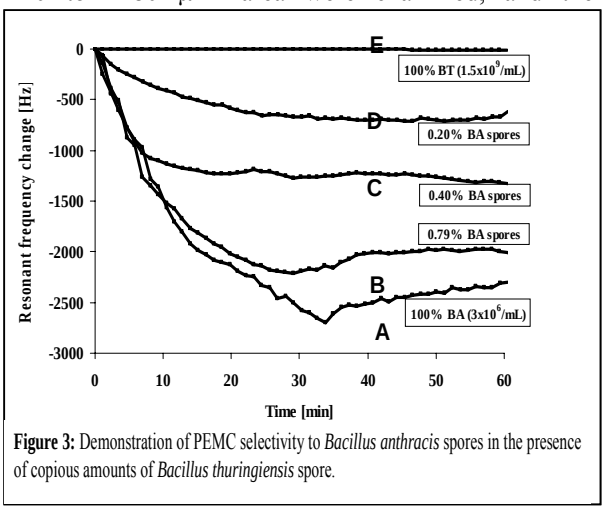


**Figure 3:** Demonstration of PEMC selectivity to *Bacillus anthracis* spores in the presence of copious amounts of *Bacillus thuringiensis* spore.

### Selective binding of Bacillus anthracis spores to antibody functionalized PEMC sensor

The sensor selectivity to *Bacillus anthracis* (BA) spore was investigated by examining the response to mixed samples containing *Bacillus thuringiensis* (BT) spores. Figure 3 shows the second flexural mode resonant frequency responses to the various mixed samples. The 100% BA spore sample (3 x $10^6$/mL; Sample A) gave the highest frequency change (2360 Hz; n=1) in 1 h. As BT spores were added to the pure BA spore sample (Samples B, C and D) the resonance frequency reached a lower steady state value: 1980 ± 10, 1310 ± 10, and 670 ± 10 Hz for the Samples B, C and D, respectively. The fluctuation in resonant frequency of the final steady state value was ± 10 Hz. It is to be noted that the pure BT spore sample (1.5 x $10^9$/mL) gave only a 10 Hz change over a 1 h period indicating that non-specific binding, if any, was weak and was insignificantly small. Sample D contained 1.5 x $10^6$ BA spores/mL and therefore, one would expect a higher resonant frequency response in comparison to the pure *Bacillus anthracis* spore sample of 3 x $10^4$ BA spores/mL (Figure 2, Panel A). However, the opposite result was observed. That is, the pure BA sample (3 x $10^4$ BA spores/mL) gave a much higher overall resonant frequency change (1030 Hz) than the Sample D containing 7.5 x $10^8$ /mL BT spores (670 ± 10Hz). These results suggest that the presence of large amount of BT spores hindered attachment of BA spores, and also reduced binding capacity. We hypothesize that BT in these experiments "crowd" the sensor surface due its very high relative concentration, which in effect reduces availability of binding sites for BA. Current work in our laboratory attempts to develop a better understanding of this phenomenon, and will be the focus of a future publication. From the results presented above, it is reasonable to conclude that the antibody functionalized PEMC sensors are selective to the *Bacillus anthracis* spores, but at a compromised sensitivity due to the presence of high BT spores.

## Kinetics of antibody and spore binding

Antibody-antigen binding on PEMC sensors has been shown to obey Langmuir kinetics.[10] At time zero, $\tau$=0, there are no concentration gradients, and thus diffusion effects are absent. Furthermore, the bulk spore concentration is known accurately. Therefore, we consider here an initial rate analysis by fitting the antibody and spore frequency response data to *Langmuir* model. The model is summarized as $(\Delta f) = (\Delta f_\infty)(1 - e^{-k_{obs}\tau})$, where $(\Delta f)$ is the change in resonant frequency at time, $\tau$, and $(\Delta f_\infty)$ is the steady state resonant frequency change. The above can be rearranged as $\ln\left(\frac{(\Delta f_\infty) - (\Delta f)}{(\Delta f_\infty)}\right) = -k_{obs}\tau$. $k_{obs}$, observed binding rate constant, can be determined during initial time (far from equilibrium) by plotting the left hand side (LHS) versus $\tau$ of the rearranged equation. where we include only data obtained during the first few minutes.

### Kinetics of antibody reaction on aminated PEMC sensor surface

Since the reaction of antibody to the aminated sensor surface is through covalent bond formation, and experimentally desorption of the bound antibody was not observed in the frequency response, it may be assumed that antibody binding was irreversible. $k_{obs}$ was determined from the data presented in Figure 1. It is clear from Figure 1 (Panel A) that the observed binding rate of gPC was significantly slower than rPC. Fitting the data to the Langmuir model gave observed binding rates of

3.6 x $10^{-2}$ $min^{-1}$ for rPC and 1.8 x $10^{-2}$ $min^{-1}$ for gPC. The antibody rPC appears not only to have attached one-third more rapidly than gPC but also the total amount bound to the sensor surface was three-fold higher.

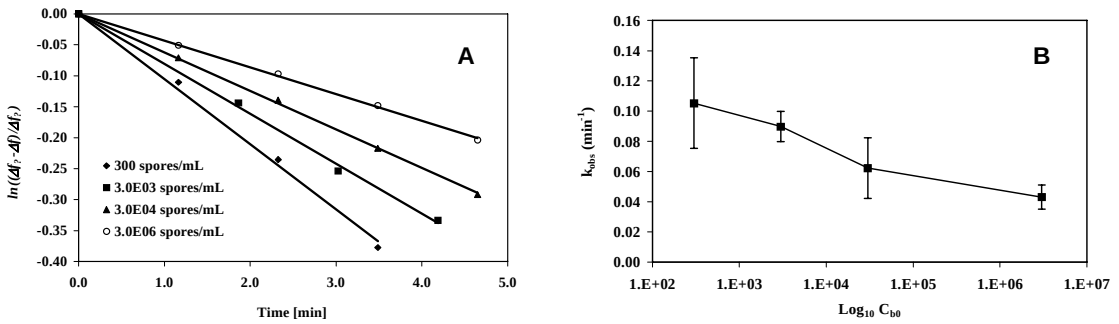


**Figure 4:** *Langmuir* kinetic analysis for the binding of *Bacillus anthracis* (BA) spores from pure samples to the PEMC sensor surface. **Panel A:** The initial kinetic analysis of the various BA spores concentrations. **Panel B**: A plot of $k_{obs}$ versus the log of the spore bulk concentrations.

**Kinetics of BA binding in pure samples**

Fitting of the experimental data presented in Figure 2 (Panel A) to the model gave straight lines with excellent correlation coefficients ranging from 0.96 to 0.99, and is illustrated in Figure 4 (Panel A). Limiting the initial rate analysis to the first five minutes appears to be a reasonable approach to obtain kinetic constants. The values of $k_{obs}$ are listed in Table 1. Here, one can see that $k_{obs}$ decreases from 0.105 $min^{-1}$ at 300 spores/mL to 0.043 $min^{-1}$ at three million spores/mL. A plot of $k_{obs}$ versus log($C_{b0}$), given in Figure 4 (Panel B) suggests that the observed rate constant is a very weak function of $C_{b0}$. Note that the decrease in $k_{obs}$ is a factor of two for a spore concentration, $C_{b0}$, change of four orders of magnitude. The results in Figure 4 (Panel B) suggest that the overall binding rate may be independent of spore concentration in the concentration range investigated. Further studies are warranted to examine the kinetics of attachment at very low spore concentration.

**Table 1:** $k_{obs}$ values as a function of *Bacillus anthracis* (BA) spore concentration in both pure and mixed samples containing BA and BT.

| BA Spores concentration (spores/mL) | BT Spores concentration (spores/mL) | $k_{obs}$ values in pure samples, PEMCa ($min^{-1}$) | $k_{obs}$ values in mixed samples, PEMCb ($min^{-1}$) |
|---|---|---|---|
| $3x10^2$ | 0 | 0.105±0.03 | ------- |
| $3x10^3$ | 0 | 0.102±0.01 | ------- |
| $3x10^4$ | 0 | 0.062±0.02 | ------- |
| $1.50x10^6$ | $7.5\ x10^8$ | ------- | 0.098±0.01 |
| $2.01x10^6$ | $5.0\ x10^8$ | ------- | 0.111±0.04 |
| $2.40x10^6$ | $3.0\ x10^8$ | ------- | 0.107±0.02 |
| $3.00x10^6$ | 0 | 0.043±0.008 | 0.067±0.01 |

**Binding kinetics of BA spores in the presence of Bacillus Thuringiensis spores**

When spores other than BA or other particulate matter are present in the sample, we expect the attachment kinetics to be affected. To what extent same fashion as the pure BA sample and a summary of the

analysis is given in Table 1. Again, the characteristic observed rate, $k_{obs}$, decreases with increasing BA spore concentration in the mixed samples. The kinetic rate analysis suggests that the presence of BT spores slightly hinders BA spores in reaching the sensor surface.

the binding rate is affected can be determined by examining the binding rate constant values when non-BA spores are present. The experimental data of the mixed samples (BA and BT) were analyzed in the

## CONCLUSIONS

In this paper we have shown that the piezoelectric excited millimeter-sized cantilever (PEMC) sensors functionalized with antibody to *Bacillus anthracis* (BA) spores can be used under liquid immersion conditions to detect in real time 300 BA spores/mL within 20 minutes. The selectivity of the antibody functionalized PEMC sensor was determined with BA samples mixed with *Bacillus thuringiensis* spore (BT). As the amount of BT spore increased in the mixed sample the steady state resonant frequency change decreased. Samples containing pure BT ($1.5 \times 10^9$/mL) gave a total frequency change of 10 Hz in 1h, which is within the noise level of the sensor. This suggests that the antibody-functionalized sensor was highly selective to the targeted pathogen. The presence of BT spores hinders BA attachment, and thus attenuates the sensor performance. The attachment kinetics of BA to the sensor surface was modeled with Langmuir model. The observed binding rate constant ranged from 0.105 to 0.043 $min^{-1}$ in the spore concentration range of 300 to 3 million per mL. These results suggest that the PEMC sensor is a viable practical sensor for BA spores in liquid medium, and in presence of BT spores.

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